

THREAT ASSESSMENT OF DISEASES OF MILITARY IMPORTANCE IN THE TROPICS

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1. Epidemiology of Enterotoxigenic *E. coli* in Thailand : Application of a Colony DNA Hybridization Assay to Detect These Pathogens

PROBLEM : To define the epidemiology of enterotoxigenic *E. coli* (ETEC) with a DNA hybridization assay.

PROGRESS : AFRIMS has recently established and tested the DNA hybridization assay to detect ETEC in diarrheal stools (1) and water (2). Four populations have been studied in the environment. A pig farm in Sri Racha has been screened to determine if pigs are a source of ETEC for man. Two villagers are being surveyed daily over one year to define the importance of ETEC as a cause of diarrhea and environmental sources of these pathogens. Over 200 children who are seen with diarrhea at Children's Hospital, Bangkok and over 900 patients with diarrhea seen at Soongnern Hospital, Soongnern have been investigated. Homes of diarrhea patients and control homes have been examined with the DNA hybridization assay.

FUTURE OBJECTIVES : The two year long study of the villages is in its eighth month and the Soongnern Hospital is in its tenth month; other populations will be examined to further determine sources of ETEC.

A new ST probe which detects ST which is only detected in pig loops will be used to determine the enteropathogenicity of this organism for man.

2. Isolation and Characterization of *Haemophilus ducreyi* in Thailand

PROBLEM : Chancroid is the second most common venereal disease in Asia (3). Previous attempts to isolate *Haemophilus ducreyi* from penile ulcers have been unsuccessful and therefore it has been impossible to determine the *in vitro* sensitivity of this organism so that rational antimicrobial therapy can be given (4).

PROGRESS : In the last two years we have been able to isolate these organisms from only three of over 300 patients. In the last two weeks we have improved our methods and are now isolating *Haemophilus ducreyi* from 60 percent of men with penile ulcers. Our increased success is due to two factors. One, we have included deactivated fetal calf serum in rabbit and horse blood in Gibco G-C base agar. Two, we have managed to reduce the fungal and bacteria contamination on the plates. Sodium azide has been included in water used to create an atmosphere with high humidity and candle jars have been washed daily with disinfectant. Normal saline used to moisten swabs was also frequently contaminated with gram negative organisms. Sterile bottles of normal saline are currently changed daily.

FUTURE OBJECTIVES : To study 100 patients with penile ulcers and culture them for *H. ducreyi*. Darkfield examination and VDRLs could also be performed to rule out syphilis and Dr. Chaninthorn of Thai Component will culture patients for *Herpes simplex*. This study should determine what proportion of penile ulcers are associated with *H. ducreyi* in Bangkok.

Isolates of *H. ducreyi* will be tested for antibiotic sensitivities to various antibiotics. Plasmids coding for antibiotic resistance will also be characterized.

Chromosomal DNA has been purified from an isolate of *H. ducreyi* and will be nick translated to incorporate α -³²P. This DNA will be used as a probe to identify this organism in pus collected from penile ulcers smeared and fixed on nitrocellulose paper. This method will be compared to routine culturing. Hopefully a method of labelling such a probe with fluorescence will have been developed in the next year which may lead to a simple rapid method of identifying *H. ducreyi*.

3. Mosquito Cytogenetic, Electrophoretic and Cross Mating Studies

PROBLEM : To define and delimit the taxa in the vector *Anopheles* species complexes by cytogenetic, electrophoretic and cross mating techniques for the following reasons : (a) to check against current morphological species concepts; (b) as an accurate determination of chromosomal polymorphisms and genetic variations in natural populations of the vector species with habitat differences, innate susceptibility to the human malarias, and behavioral patterns that may facilitate more effective control measures in the future.

PROGRESS : Preliminary analysis of salivary gland chromosomes reveals that the autosomes of *Anopheles dirus* (5), *An. takasagoensis*, *An. balabacensis* Perlis form and Fraser's Hill form appear to be very similar. Nevertheless, the X chromosome seems to be different in length, with different banding sequences in zone 6. Analyses of mitotic and meiotic karyotypes of these species and forms of the *An. balabacensis* complex show that metaphase chromosomes, especially the sex chromosomes of *An. dirus*, *An. takasagoensis* and *An. balabacensis* Perlis form are significantly different. These karyological differences are very useful in differentiating these taxa, particularly the Perlis form, and support the concept of these taxa being elevated to species status (6).

An attempt to find biochemical markers for interspecific differences between *An. dirus*, *An. balabacensis* Perlis form and Fraser's Hill form has been carried out using starch and polyacrylamide gel electrophoretic techniques. The latter seems to yield better resolution. In the Balabacensis complex, esterases 2 and 3 show polymorphic bands with fast, intermediate, and slow electromorphs for each locus. Differences were noted between the members of the complex when testing for the enzymes ODH, XDH, and PGI-1.

Forced mating crosses in both directions between *An. balabacensis* Perlis form and Fraser's Hill form produced only small numbers of viable F1 hybrid offspring in one direction and no viable offspring in the other direction. In contrast, crosses between Fraser's Hill form and *An. dirus* produced normal hybrid offspring through the 3rd generation.

These cytological, electrophoretic, and crossing data, together with morphological studies, clearly indicate that *An. balabacensis* is actually a sibling species complex consisting of at least four full biological species. Similarly, preliminary results of studies on *An. maculatus* and *An. nivipes* (7) show that there are at least three and two sibling species of these mosquitoes, respectively, in Thailand.

FUTURE OBJECTIVES : These studies on the sibling species complexes of vector anophelines in Thailand will continue during the coming year. Emphasis will be placed on natural mosquito population determinations correlated with formal genetic studies using cytogenetic and electrophoretic techniques.

4. Comparative Susceptibility of Known and Suspected Species/Strains of Anopheles to Plasmodium Parasites

PROBLEM : The objectives of this investigation are as follows : (a) To determine and compare the susceptibility of primary and potential secondary vectors of malaria to *Plasmodium* parasites; (b) to delineate the development of malaria parasites in *Anopheles* spp. with varying degrees of susceptibility; and (c) to observe the feeding behavior of colonized vectors of human malaria under laboratory conditions.

PROGRESS: During this reporting period, attempts were made to find a partially refractory anopheline to *Plasmodium cynomolgi* (8) and/or *P. knowlesi*. Of eight species or strains tested, most were less susceptible than *An. dirus*, but none which developed oocysts failed to go to the sporozoite stage.

Human susceptibility studies were initiated at the start of this fiscal year in Tha Muang, Kanchanaburi. Over 75 falciparum patients and 100 vivax patients have volunteered for the study. Unfortunately, less than 40 percent of the feeds infected mosquitoes. *An. dirus* was used as the control mosquito species and was more susceptible to both *Plasmodium* species than were the other mosquito species tested. Of the experimental mosquito species, *An. balabacensis* Perlis form was the most susceptible and *An. maculatus* Huai Kuum strain the least. All species developed some infected salivary glands by Day 14.

FUTURE OBJECTIVES: Due to the high number of negative feeds and the number of species/strains of mosquitoes left to be tested, this study will continue for at least another year. Attempts to correlate negative feeds with seasonality or prior drug usage will be made.

5. Identification of Field-Collected Sporozoites

PROBLEM : Three different tests are available to potentially identify *Plasmodium* sporozoites in mosquito salivary glands: (1) circum-sporozoite precipitin test; (2) immunofluorescent antibody test; and (3) radioimmuno assay test. The objective of this study is to evaluate these three tests in the laboratory, and then adapt the tests to use in the field to identify natural infections in vector anophelines.

PROGRESS : Laboratory-reared mosquitoes were fed on patients infected with gametocytes of *Plasmodium vivax* or *P. falciparum*. Other mosquitoes were fed on monkeys infected with *P. cynomolgi* or *P. knowlesi*. Infected salivary glands were later crushed, and the sporozoites were inoculated into rabbits for antibody production. At least four boosters were given to each rabbit over a period of several months. Known sporozoites were added to antiserum samples, and then were examined microscopically following incubation. The circum-sporozoite precipitin reaction (9) was readily visible with some sporozoites and appeared to be species specific.

FUTURE OBJECTIVES : During the next year, the immunofluorescent antibody test and the radioimmuno assay test will be evaluated in collaboration with NIH. An attempt will be made to also develop an ELISA test for use in the field.

6. Mosquito Survey and Taxonomic Studies

PROBLEM : To elucidate the mosquito fauna of Thailand and Southeast Asia. Primary emphasis is put on the determination of diagnostic characters that separate the vector species and groups containing vector species that transmit parasite detrimental to humans.

PROGRESS : Morphological studies on the *Leucosphyrus* Complex (10), the *Maculatus* Complex, and the *Anopheles philippinensis-nivipes* group were continued during the past year. A manuscript confirming a separate species status for *An. philippinensis* and *An. nivipes* has been cleared for publication (11). Work on the taxonomic revision of the Kochi group of *Aedes* (*Finlaya*) continued. Descriptions and keys for this group have been completed and manuscript prepared; however, many illustrations of the various stages of these species remain to be done. The surveillance of vector species densities and distributions in Thailand and Malaysia revealed the potential of several important vector-human parasite interactions. The guide to the genera of mosquitoes occurring in Thailand, along with illustrated keys, biological notes, and mounting techniques, was revised and is now in press.

FUTURE OBJECTIVES : During the next year primary emphasis will be placed on delineating usable morphological markers to identify natural populations of

the sibling species complexes of *An. balabacensis* and *An. maculatus*. Illustrations for the *Aedes* (Finlaya) manuscript will be finalized. Preparation of keys specific to human disease vectors in Southeast Asia is proposed.

7. Detrimental Effects of Plasmodium Infections on the Survival Rate of *Anopheles dirus*

PROBLEM : The objectives of this study are as follows: (a) to determine if the longevity of mosquitoes infected with *Plasmodium* is different significantly from that of uninfected mosquitoes; (b) to determine if the longevity among mosquitoes with heavy or light infection rates is significantly different; and (c) to determine if the longevity of mosquitoes infected with different species of *Plasmodium* is different significantly among groups.

PROGRESS : Manuscripts describing the results of studies on the effects of *Plasmodium cynomolgi* on *Anopheles dirus* were revised this year and sent for publication. The first manuscript covers differences found in the mortality rates of non-infected versus infected groups, and this manuscript has appeared in print (12). The correlation of differences in the survival rates of mosquitoes infected with various densities of parasites is discussed in the second manuscript which is in press (13).

An investigation of the effects of human malaria parasites on the longevity of *An. dirus* was begun this year. One hundred lots of mosquitoes have been fed on vivax patients and 75 on falciparum patients. Only 40 percent of the feeds were infective to the mosquitoes. Lightly infected mosquitoes and controls live two months; whereas, many of the heavily infected mosquitoes died during the first 30 days. These studies will continue for another year so that the results can be statistically analyzed.

8. Serosurvey and Virus Isolation from Rodents to Determine the Hantaan Virus Presence in Thailand

PROBLEM : Recent studies have found Hantaan virus to be the causative agent of Korean hemorrhagic fever (KHF), a syndrome of significance in Korea and Manchuria and of potential significance in the USSR, the Balkans, parts of Western Europe and Scandinavia (14). Evidence has recently been obtained in Seoul, Korea that urban *Rattus* also are chronically infected with Hantaan virus. Cases of KHF in man have now been linked temporarily and geographically to infected wild rats in urban Seoul and Osaka, Japan (15). In addition, antibodies to Hantaan virus have been found in *Rattus* captured near the docks in Japan, Korea and the United States. Chronic infection, rats and international shipping thus provide a likely chain which may have disseminated this virus worldwide. Thus the potential for this agent to cause human disease may be far greater and more geographically diverse than is presently appreciated.

The objectives of this study are (1) Identify areas of Thailand where Hantaan virus antibody positive rodents exists and test human sera in those areas; (2) isolation of Hantaan virus from infected rodents.

PROGRESS : In a collaborative effect with MAJ LeDuc, USAMRIID, Ft. Detrick, a serosurvey of 100 rodents was completed at the Klong Toey port in Bangkok. Five positive sera were found and four of these were from one sepcific area at the port. A follow up trapping of rodents at this location has been completed and tissue samples have been frozen for Hantaan virus isolation at USAMRIID. Serum samples are currently being collected from people working in the vicinity of the rodent trapping area.

FUTURE OBJECTIVES : A serosurvey and tissue collection for virus isolation from rodents will begin next month at the Bang Pacong port area to determine if Hantaan virus is present there.

9. Epidemiology of Encephalitis in Thailand

PROBLEM : Encephalitis in general, and Japanese B encephalitis (JE) in particular, have been recognized as diseases of major epidemiological risk in Thailand. In 1969 and 1970, a detailed analysis of JE in Northern Thailand was performed, but no review has been done since then. A primary goal of field investigation teams is to supply information which is directly useful in the control of disease. Commitment of resources can be directed only by detailed knowledge of the epidemiological importance of a disease. Therefore, encephalitis surveillance data collected by the Center for Disease Control of Thailand has been reviewed in order to ascertain the population most likely to benefit from attempts to control JE.

PROGRESS : Reported numbers of cases of encephalitis are tabulated by the Center for Disease Control and population data by the Department of Vital Statistics. 1970 census data was used for estimation of age specific rates. Overall, an average of 1598 cases (range = 986 in 1970 to 2413 in 1980) were reported each year, with an average case fatality ratio of 23 percent. A general upward trend in reported cases has occurred. In each year the peak number of cases occurred in July, though in three years the peak encompassed two months (July plus either June or August). Cases were reported in all months. Each year, the Northern region contributed the largest number of cases (34 percent on the average). Within the Northern region, the provinces of Uttaradit (46 cases/100,000), Phayao (21), Chiang Rai (19), Chiang Mai (11), and Nan (26), reported the highest rates in 1980. The lowest rates have been from Southern provinces. Age specific attack rates were about 30/100,000 in children age 1-17 in Uttaradit, and about 18/100,000 in similar age groups in Chiang Mai. These data show that the northern region remains the area of highest risk for encephalitis, and that certain provinces consistantly report higher rates than others. Children under 14 are at highest risk. Risk estimates are consistant with earlier published data.

FUTURE OBJECTIVES :

1. Further study should be under taken to ascertain the proportion of encephalitis which is due to JE.
2. If substantial numbers of cases are not due to JE, efforts should be directed at determining actual etiology.

3. Programs to evaluate measures to reduce JE transmission should be undertaken. These surveillance data can be used to effectively guide such studies.

10. Acute Encephalitis at Bangkok Children's Hospital

PROBLEM : Acute encephalitis contributes a large number of cases each year to the patient load at Bangkok Children's Hospital (16) and has been a threat to American soldiers stationed in Southeast Asia (17). To assess the magnitude of encephalitis as a problem, determine the flavivirus etiology of such cases, and ascertain the population at risk, surveillance of encephalitis has been maintained at Children's Hospital for four years.

PROGRESS : Since 1979, all cases with encephalitis have been seen and interviewed. Clinical and epidemiological data, paired sera and cerebrospinal fluid (CSF) were collected and tested using HAI for flaviviruses and IgM and IgG capture (MAC and GAC) ELISAS for Japanese B encephalitis (JE) (18). Diagnosis of JE required a four fold HAI rise or a MAC ELISA OD reading of greater than the average reading for a known weak positive specimen in either serum or CSF.

Eighty-three cases of encephalitis were seen at Children's Hospital between January 1979 and September 1982. Fifty-eight percent of these were due to Japanese B encephalitis by either test, an average of 12 cases per year. Twenty percent of JE cases and seventeen percent of Non-JE cases were fatal during the initial hospital admission. Thirty-four percent of the JE cases were male. Their mean age was 6.9 years (range one year to 14 years). Most cases came from the provinces surrounding Bangkok although 27 percent had Bangkok addresses. Cases occurred between March and December. No cases were seen in January or February, while 33 percent of cases occurred during June (eight percent expected) the largest percentage of any month. Agreement between HAI and ELISA diagnosis occurred in 46 of the JE cases completely studied (20 JE and 26 not JE). Approximately 50 percent of encephalitis was not due to JE or dengue. No cases were HAI positive and ELISA negative. However, ten cases were ELISA positive and HAI negative. Analysis of these ten cases revealed that six had stable HAI titers of ≥ 80 . Of these five had anti-JE IgM in both sera. Three had anti-JE IgM in CSF and the other three were not tested. These six were probably due to JE. By contrast, the other four of the ten patients with conflicting results were HAI seronegative but had a single serum (3) or two sera (1) positive for anti-JE IgM. All three CSF's tested from these four patients were negative for anti-JE IgM. These four cases may have been infected by JE, but their encephalitis was probably of another etiology, pointing out the importance of studying CSF by ELISA before diagnosing JE encephalitis. Only one patient had a serum ELISA positive for dengue, and he had a broad 2^o flavivirus response. Thus, one case of encephalitis may have been due to dengue virus.

FUTURE OBJECTIVES :

1. Continue surveillance of encephalitis seen at Children's Hospital.
2. Attempt to ascertain the etiology of Non-JE encephalitis.

3. Should anti-viral chemotherapy, such as ribavirin, prove promising in animal models, design protocols to evaluate its use in treating human cases.

11. Dengue Hemorrhagic Fever and Pyrexia of Unknown Origin at Children's Hospital

PROBLEM : Previous studies have linked pre-existing dengue antibody with the occurrence of hemorrhagic fever and shock during second infections. This hypothesis has important implications not only for understanding the pathogenesis of hemorrhagic fever, but also for the development of dengue vaccines. Also, pyrexia, undiagnosed on admission (PUO) been shown to be frequently due to dengue infection in Both Thai children and U.S. soldiers stationed in Southeast Asia. Finally, surveillance of dengue hemorrhagic fever (DHF) and PUO's at Children's Hospital in Bangkok has been maintained to study these conditions. Data collected in Bangkok during the previous 20 years has shown that the four dengue virus types do not cause DHF or dengue fever with the same frequency and do not appear in the community at the same time. The identification of dengue virus isolated during each year has allowed prediction of the virus type of future isolations.

PROGRESS : During calendar year 1981, 198 cases of DHF were seen at Children's Hospital. Two studies of paired sera showed that 10 percent of these were primary paired sera showed that 10 percent of these were primary and 80 percent secondary infections. Five percent were not dengue and five percent were unavailable for follow up. Thus far, virus isolates have been obtained from 32 cases. D1 was obtained in four primary and five secondary infections. D2 was found in one primary and 17 secondary infections. No virus isolates were obtained from the nine non-dengue cases. Data from 42 PUO patients seen between January and September showed that 12 percent were primary, ten percent secondary, 62 percent not dengue, and 16 percent were lost to follow up. Through September, 131 cases of hemorrhagic fever were seen in 1982. Of these 14 percent were primary dengue infection, 72 percent secondary flavivirus infection and 11 percent were not dengue infections. Only three isolates have been obtained thus far, and all are D2.

These data confirm previous data which show (1) a large fraction of PUO's are due to dengue infections (22 percent), (2) DHF is associated with a secondary flavivirus antibody response, and (3) D2 is rarely the etiology of primary DHF, but is the most commonly isolated strain from 2^o cases.

FUTURE OBJECTIVES :

1. The PUO portion of this study was complete and was terminated in September of 1981.

2. DHF surveillance will continue in order to increase knowledge of viruses of likely risk to seronegative individuals.

3. Isolation attempts will continue on remaining sera.

4. DHF surveillance should be expanded to include other centers in Southeast Asia and the Western Pacific in order to gain broader knowledge about the spread of dengue viruses throughout this region. The circulation of multiple dengue strains in the Caribbean region, the recent occurrence of DHF in that area, and of dengue in the continental United States mandate that we maximize our opportunities to learn about the spread, pathogenesis and prevention of dengue infection.

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